Roziah et al., Entomol Ornithol Herpetol 2015, 4:4
DOI: 10.4172/2161-0983.1000163

Entomology, Ornithology & Herpetology

Research Article Open Access

Mitochondrial and Nuclear DNA for Identification of Forensically Important Flesh Flies (Sarcophagidae: *Boettcherisca* Spp).

Roziah A[1,2]*, Tan SH[2], Lee HL[1] and Mohamed Z[2]

[1]*Medical Entomology Unit, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia*
[2]*Unit of Genetics and Molecular Biology, Institute Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia*

Abstract

Morphology-based identification of flesh flies (Family: Sarcophagidae) is normally limited to genus level due to their high similarities in characteristics. Even though identification to species level is possible, expertise in taxonomy is still needed; as sarcophagid female flies are extremely difficult to identify. In this study, a molecular approach was used to examine the use of *12S & 16S* ribosomal mitochondrial DNA (1172 bp) and Internal Transcribed Spacer (ITS) region of nuclear DNA (~1500 bp) to discriminate four species of the subgenus *Boettcherisca* namely, *Boettcherisca javanica, B. peregrina, B. karnyi* and *B. highlandica*. Neighbour-Joining phylogenetic tree was generated for each gene. Interspecific values calculated using the Kimura-2-parameter distance model were in the low range of 0.5%-1.3% and 0.5%-1.9% for *12S & 16S*rDNA and ITS region respectively. However, identifications of *B. karnyi* and *B. peregrina* using ITS; and *B. peregrina* using 12S & 16S alone can be ambiguous. Therefore phylogenetic tree analyses of both genes showed a likely for these specimens to be distinguished and confirmed the potential of these genes as specific sarcophagid identification markers.

Introduction

Flies have been widely used in crime scene investigation and legal cases especially in the determination of the Post Mortem Interval (PMI) [1]. Commonly reported flies from Calliphoridae, Sarcophagidae, Stratiomyidae and Muscidae families are the early colonisers that can be found on human corpse [2,3].

Flesh fly is a common name for the members of the Sarcophagidae family. The larvae of some species have been recorded from human corpses and the larvae of many species are associated with decaying or rotten materials. The adult flies are usually attracted to odours of decay or nectar [4]. In terms of life cycle, flesh fly is viviparous. They give birth to larvae (maggots) near or on the food source as the eggs hatch when they are expelled from the ovipositor. Because of their habit and reproductive cycle, the Sarcophagids can be suitably and reliably used to estimate the PMI [5].

However, to be able to use the information provided by the fly collected from the crime scene, accurate identification of the fly is the most crucial step in forensic entomology practice. Conventional morphology-based identification of Sarcophagids is notoriously difficult-especially for female flies. It is also difficult to identify the immature stages and these usually have to be reared to the adult stage. However, this step is risky because the maggot may not be able to survive during the breeding process and it is time consuming.

Molecular taxonomy has been suggested as an alternative to conventional morphology-based identification in forensic entomology [6]. It has been a valuable alternative to the conventional method due to its flexibility, accuracy and ease to conduct. In molecular approach, rearing of the immature flies is no longer necessary because only a minimal material of the fly (eggs, larvae, pupae or adults) is required. It is also applicable to preserved fly specimens. Molecular analysis can be performed by a trained laboratory worker, compared to conventional method that requires expert taxonomist to identify the flies [7].

In molecular taxonomy, several DNA regions have been explored to identify the forensically important flies. Mitochondrial DNA (mtDNA) is the most common DNA region that is able to successfully differentiate several species of the forensic flies. Because it is maternally inherited, mtDNA is also suitable in resolving the relationships of closely related species of some forensically important fly species [8-11]. Most of the previous studies mainly focused on the cytochrome oxidase subunit I & II (*COI & COII*) genes in molecular identification of forensically important fly species [12, 13]. In this study, *12S & 16S* ribosomal DNA (*12S rDNA*& *16S rDNA*) of the mitochondrial DNA were selected for analysis, because these genes have been successfully used to discriminate the species of Muscidae and Calliphoridae species [14,15]. However, according to Wells & Steven (2008) [16] molecular approach may not be accurate if relied only on one gene. Internal Transcribed Spacer Region (*ITS*) has been used in several studies for identification of forensic fly. This region has proven to be suitable for phylogenetic studies at the species and intraspecific levels because of the high evolutionary rates [8]. The utility of ITS region barcodes has been explored for the identification of 29 fly species comprise of 3 families; Calliphoridae, Muscidae and Sarcophagidae [17]. Thus, this study examines the possibility of both mitochondrial and nuclear DNA to discriminate several closely related species of Sarcophagidae family, subgenus *Boettcherisca*.

Materials and Methods

Specimens

Four species of *Boettcherisca* genus were collected from different localities (Table 1). All samples were pinned at the thorax slightly to the right and all legs were straightened down. The male genitalia was

***Corresponding author:** Roziah A, Medical Entomology Unit, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia. Tel: 603-2616 2666; Email: roziah@imr.gov.my

Received July 10, 2015; **Accepted** August 21, 2015; **Published** August 25, 2015

Citation: Roziah A, Tan SH, Lee HL, Mohamed Z (2015) Mitochondrial and Nuclear DNA for Identification of Forensically Important Flesh Flies (Sarcophagidae: *Boettcherisca* Spp). Entomol Ornithol Herpetol 4: 163. doi:10.4172/2161-0983.1000163

No	Species	Accession number		Locality	Voucher
		12S/16S rDNA gene	*ITS* region		
1	*B. javanica*	KF038379	JX219269	Botanical Garden, Kuala Lumpur	BJ11
2	*B. javanica*	KF038380		Botanical Garden, Kuala Lumpur	BJ12
3	*B. peregrina*	KF038381		Botanical Garden, Kuala Lumpur	BP8
4	*B. peregrina*	KF038382	JX219270	Gohtong Jaya, Genting Highland	BP4
5	*B. highlandica*	KF038383	JX292016	Gombak, Selangor	BH1
6	*B. karnyi*	KF038384		Pantai Morib, Selangor	BK1
7	*B. karnyi*	KF038385	JX292017	Pantai Morib, Selangor	BK3

Table 1: Information on specimens used in this study with accession number based on *12S & 16S*rDNA gene and ITS region.

pulled out and fixed in position by using pieces of paper and examined under the microscope. One or two legs from each fly were used for molecular analysis.

DNA extraction

The legs of the flies were placed in a 1.5mL micro centrifuge tube and snap frozen in liquid nitrogen. Subsequently the sample was crushed into fine powder using a sterile mini pestle. DNA extraction was carried out using QIAGEN™ DNA extraction kit (QIAGEN™, Germany) following the manufacturer's protocol provided. The DNA samples were stored at -20°C (National™) until used.

PCR and cloning

PCR amplification was carried out in 25 µL reaction mixture containing 100ng template DNA, 1 x buffer (Bio tools ™, Spain), 2mM MgCl2 (Bio tools™, Spain), 200µM of each dNTP, 20µM of each primer, 1.0U Taq Polymerase (Bio tools™, Spain) and distilled water in a sterile 0.2µL micro centrifuge tube. PCR was performed with a Peltier Thermal Cylcer™100 or Peltier Thermal Cylcer™200 (MJ Research™).

PCR amplification profile comprised an initial denaturation step at 94°C for 5 min, 35 cycles of 94°C f or 1 min, annealing temperature for 1 min 30 s, 72°C for 2 min 30 s and final elongation step at 72°C for 5 min. Annealing temperature used for each primer was determined with the PCR gradient temperature together with the primer sequences (Table 2). Target amplified regions were illustrated in Figure 1. Amplified product was subjected to agarose gel electrophoresis and purified prior to gene cloning and DNA sequencing. The purification was carried out with QIAquick™ Gel Extraction by following the manufacturer's protocol provided. The purified gel extracted DNA product was subjected to gel electrophoresis and cloned into a pGEM-T Easy Vector Systems™ (Promega™, USA) following the manufacturer's protocol.

Sequencing and DNA analysis

Sequence analyses were conducted by concatenating two contig sequences (forward and reverse) using Chromas® software version 4.0. All concatenated sequences obtained were subjected to run in Basic Local Alignment Search Tool (BLAST®) retrieved from (http://blast.ncbi.nlm.nih.gov/) available in the GenBank

Sequences showing high similarity were then aligned using Clustal W program available in Molecular Evolutionary Genetics Analysis (MEGA®) 5.1 software. Sequences were aligned according to the species and DNA region to resolve any insertion, deletion or substitution of the nucleotide. Nucleotide composition and sequence divergence were then calculated using the same software.

Finally, neighbour-joining tree analysis was constructed and the results of all DNA regions were compared. The tree was constructed using Kimura-2-parameter model and bootstrap value of 1000 replicates. A sequence from *Roselia notabilis* species of Sarcophagidae family was used as an outgroup to each DNA region analysed.

Result and Discussion

A total of 1173 bp of *12S & 16S* rDNA region and approximately ~1500bp of ITS regions were obtained from all individual fly. All sequences were submitted to GenBank (NCBI) (Table 2). No insertion or deletion was detected from the *12S &16S* rDNA gene sequences. However, the amplification of ITS regions varied in length for each species: *Boettcherisca javanica* (1540bp), *Boettcherisca peregrina* (1551bp), *Boettcherisca karnyi* (1550bp), and *Boettcherisca highlandica*(1558 bp). These findings showed consistency with previous data [7,8] reported that PCR products amplified from ITS gene using the same set of primer varied and ranged from 1.2kb to 1.5kb for forensic flies species. On the other hand, the sizes of PCR product from the same species were in the same length and supported the findings by previous study [7] who stated that amplification of ITS region from *Ch. incisuralis* with the same primer showed similar length, indicating lesser intraspecific differences of *ITS* gene from these species.

Seven specimens of *Boettcherisca* subgenus and *Roselia notabilis* as an outgroup were included in phylogenetic analyses for both DNA regions. Two neighbour-joining trees were constructed based on the*12S&16S* rDNA and ITS genes with the bootstrap value of 1000 replicate (Figures 2 and 3). Phylogenetic analysis performed based

DNA region	Primer ID & sequences	Annealing temperature
*12S &16S*rDNA	mtD-33 forward - ATGTTTTTGTTAAACAGGCG mtD-12S reverse - AAACTAGGATTAGATACCCTATTAT	60°C
ITS region	18SF-1975 forward - TAACAAGGTTTCCGTAGGTG 28SR-52 reverse - GTTAGTTTCTTTTCCTCCCCT	62°C

Table 2: Primer sequences and annealing temperatures used to amplify two different DNA region.

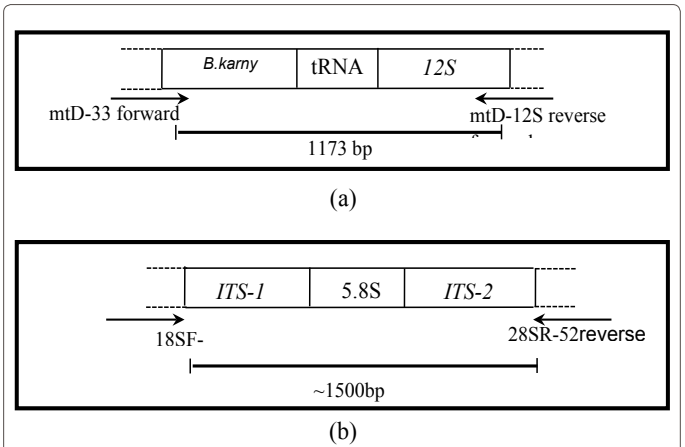

Figure 1: Schematic representation of amplified (a) *12S & 16S* segments of mitochondrial DNA and (b) ITS regions. Locations of the primers and sizes of the amplification fragments using different primer combinations are shown.

on *12S&16S* rDNA region showed that different branch was formed with high bootstrap support for *Boettcherisca javanica, Boettcherisca karnyi* and *Boettcherisca highlandica*. However, *Boettcherisca peregrina* specimens were not grouped together in the same clade. BP8 specimen was in the same clade with *B. javanica* species, whilst BP4 specimen was with the same clade with *B. karnyi* with the support value of 60% and 94% respectively. Even though this region was able to place *B. peregrina* in the same group, monophyletic separation of other species in the phylogenetic tree confirmed that this gene was an effective genetic marker. Furthermore, pairwise distance values for interspecific and intraspecific relationships were calculated (Table 3). The highest and lowest pairwise distance value for the same species obtained were 1.1% and 0.3% for *B. peregrina* and *B. karnyi* respectively. The levels of interspecific variations based on this gene ranged from 0.5%-1.3% for BP4 & BK3 and BK4 & BK11, respectively. Maximum intraspecific value obtained from this region is still in the ideal range of intraspecific variation (<3%) [5]. However, the small range of the interspecific value in this study could indicate that the specimens are very closely related under the same subgenus.

Phylogenetic analysis tree based on *ITS* regions formed two single clades with the support value of 91% (*B. highlandica, B. peregrina* and *B. karnyi*) and 51% (*B. javanica*). Even though *Boettcherisca karnyi* samples were not grouped together, they still formed a separate branch from other species. The separation of *B. highlandica* (BH1) specimen with high bootstrapped value (91%) showed that this gene was as good as *12S &16S* rDNA gene to distinguish it from other species. In the analysis of pairwise distance, there are minor differences of intraspecific values from all species. The value ranged from 0.3% - 0.8% and was mainly due to pair of *B. peregrina* and pair of *B. karnyi* specimens respectively. The small value was similar to the analysis based on the *12S & 16S*rDNA gene because of closely related species.

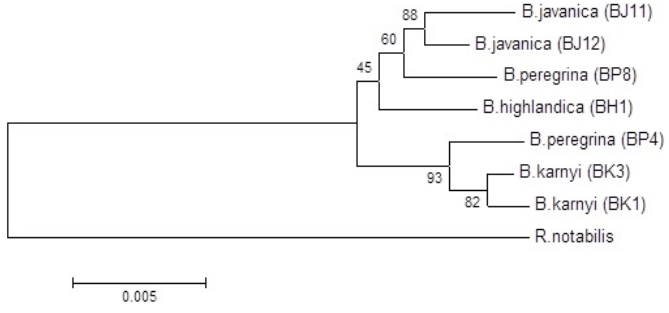

Figure 2: Neighbour-joining tree of *12S & 16S rDNA* gene with bootstrap support.

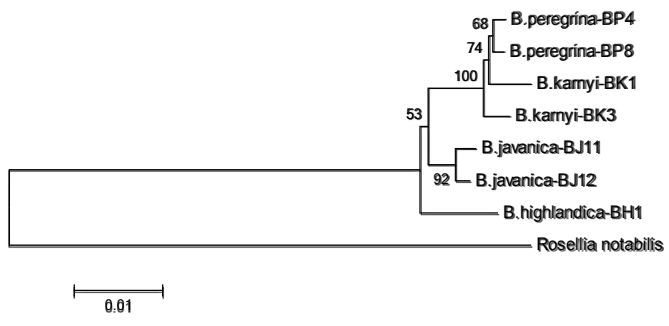

Figure 3: Neighbour-joining tree of ITS region with bootstrap support.

		[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]
[1]	*B. javanica* (BJ11)		0.004	0.017	0.019	0.013	0.017	0.017	0.106
[2]	*B. javanica* (BJ12)	0.005		0.016	0.018	0.012	0.016	0.016	0.107
[3]	*B. karnyi* (BK3)	0.012	0.010		0.008	0.017	0.005	0.005	0.117
[4]	*B. karnyi* (BK4)	0.013	0.011	0.003		0.019	0.006	0.006	0.120
[5]	*B. highlandica* (BH1)	0.009	0.007	0.010	0.011		0.017	0.017	0.113
[6]	*B. peregrina* (BP4)	0.012	0.010	0.005	0.006	0.010		0.003	0.118
[7]	*B. peregrina* (BP8)	0.008	0.006	0.011	0.012	0.009	0.011		0.117
[8]	*Rosellia notabilis*	0.039	0.037	0.039	0.038	0.038	0.040	0.038	

Table 3: (Lower left) Pairwise distance matrix of *12S &16S rDNA* gene. (Upper right) Pairwise distance matrix of ITS region.

In the interspecific variation analysis, the variations were also small and ranging from 0.5% - 1.9%. The lowest value was obtained from the pair of both specimens of *B. peregrina* & BK3 (0.5%), whilst the highest value was from pair of BK4 & BH1 specimens (1.9%).

From the phylogenetic analysis, *B. peregrina* could not be separated using 12S & 16S rDNA, but well grouped in the same clades by ITS regions. There was also minor difference in terms of pairwise variations analysis between *12S & 16S* rDNA gene and *ITS* region. Interspecific values for both genes were small and not in the ideal range to distinguish between species. This could be because all species are in the same subgenus (*Boettcherisca*) and some of it are in the same group (*peregrina*-group) such as *B. peregrina* and *B. javanica* (Tan *et al.*, 2010). However, the present study was not able to separate the species according to the geographical location. This was concordance with the study reported previous study [5,14,15] that the samples were grouping into the same species regardless of the location collected.

In this study, we assessed *12S & 16S* rDNA and ITS region as potential markers to identify the closely related species of forensically important flies under subgenus of *Boettcherisca* (Sarcophagidae). Phylogenetic analyses showed that there are differences between *12S & 16S* rDNA and ITS region cladogram. Regardless of the low interspecific values variation, *B. javanica, B. highlandica* and *B. karnyi* species can be separated clearly in *12S & 16S* rDNA phylogenetic analysis. The ITS region analysis demonstrated that *B. peregrina* species can be grouped in the same clade. These findings supported previous studies that there was no single gene that could be relied on to discriminate the species of the forensically important flies [16,19].

The use of molecular marker for identification could be more effective if both genes were used. Future study with more specimens from the same genus and different geographical areas could improve the phylogenetic resolution.

Acknowledgements

The authors thank the Director General of Health, Malaysia for permission to publish and Director, Institute for Medical Research, Kuala Lumpur for support.

References

1. Catts EP, Goff ML (1992) Forensic entomology in criminal investigations. Annu Rev Entomol 37: 253-272.
2. Heo CC, Mohamad AM, Ahmad Firdaus MS, Jeffery J, Baharudin O (2007) A preliminary study of insect succession on a pig carcass in a palm oil plantation in Malaysia. Trop Biomed 24: 23-27.
3. Nazni WA, Lee HL, Chen CD, Chin HC, Abdullah AG, et al. (2011) Comparative insect fauna succession on indoor and outdoor monkey carrions in a semi-

forested area in Malaysia. Asian Pacific Journal of Tropical Biomedicine S232-S238.

4. Byrd JH, Castner JL (2010) Forensic Entomology: The utility of arthropods in legal investigations (Second Edition). United States: Taylor and Francis.
5. Meiklejohn KA, Wallman JF, Dowton M (2011) DNA-based identification of forensically important Australian Sarcophagidae (Diptera). Int J Legal Med 125: 27-32.
6. Sperling FA, Anderson GS, Hickey DA (1994) A DNA-based approach to the identification of insect species used for postmortem interval estimation. J Forensic Sci 39: 418-427.
7. Nelson LA, Wallman JF, Dowton M (2008) Identification of forensically important Chrysomya (Diptera: Calliphoridae) species using the second ribosomal internal transcribed spacer (ITS2). Forensic Science International, 177: 238-247.
8. Hillis DM, Moritz M, Mable BK (1996) Molecular Systematic (Second Edition). Sunderland, Massachusetts USA. Sinauer Associates, Inc.
9. Tan SH, Mohd Aris E, Surin J, Baharudin O, Kurahashi H (2009) Sequence variation in the cytochrome oxidase subunit I and II genes of two commonly found blow fly species Chrysomya megacephala (Fabricius)and Chrysomya rufifacies (Macquart)(Diptera: Calliphoridae) in Malaysia. Tropical Biomedicine 26: 173–181.
10. Tan SH, Rizman-Idid M, Mohd-Aris E, Kurahashi H, Mohamed Z (2010) DNA-based characterisation and classification of forensically important flesh flies (Diptera: Sarcophagidae) in Malaysia. Forensic Sci Int 199: 43-49.
11. Kavitha R, Tan TC, Lee HL, Nazni WA, Sofian AM (2013) Molecular identification of Malaysian Chrysomya megacephala (Fabricius) and Chrysomya rufifacies (Macquart) using life stage specific mitochondrial DNA. Trop Biomed 30: 211-219.
12. Zehner R, Amendt J, Schütt S, Sauer J, Krettek R, et al. (2004) Genetic identification of forensically important flesh flies (Diptera: Sarcophagidae). Int J Legal Med 118: 245-247.
13. Wells JD, Pape T, Sperling FA (2001) DNA-based identification and molecular systematics of forensically important Sarcophagidae (Diptera). J Forensic Sci 46: 1098-1102.
14. Li X1, Cai JF, Guo YD, Wu KL, Wang JF, et al. (2010) The availability of 16S rRNA for the identification of forensically important flies (Diptera: Muscidae) in China. Trop Biomed 27: 155-166.
15. Guo Y, Cai J, Chang Y, Li X, Liu Q, et al. (2011) Identification of forensically important sarcophagid flies (Diptera: Sarcophagidae) in China, based on COI and 16S rDNA gene sequences. J Forensic Sci 56: 1534-1540.
16. Wells JD, Wall R, Stevens JR (2007) Phylogenetic analysis of forensically important Lucilia flies based on cytochrome oxidase I sequence: a cautionary tale for forensic species determination. Int J Legal Med 121: 229-233.
17. Song ZK, Wang XZ, Liang GQ (2008) Species identification of some common necrophagous flies in Guangdong province, southern China based on the rDNA internal transcribed spacer 2 (ITS2). Forensic Science International. 175: 17–22.
18. Ratcliffe ST, Webb DW, Weinzievr RA, Robertson HM (2003) PCR-RFLP identification of Diptera (Calliphoridae, Muscidae and Sarcophagidae)--a generally applicable method. J Forensic Sci 48: 783-785.
19. Zaidi F, Wei SJ, Shi M, Chen XX (2011) Utility of multi-gene loci for forensic species diagnosis of blowflies. J Insect Sci 11: 59.